recruitment of the circulating cells expressing CXCR4 into the injured myocardial territory to contribute to healing and regeneration of the tissue. This CXCR4–SDF-1 axis may also be a target to enhance IC cell injection [80]. Further preclinical optimization is essential for these treatments to be applicable in the clinical arena.

Enhancing survival of retained donor cells is also a strategy to enhance donor cell engraftment after IC cell injection. For this aim, the strategies to improve donor cell survival after IM injection, which are discussed in the section of IM cell injection, are similarly applicable, although these have not been studied in the scenario of IC cell injection.

Use of the retrograde IC route is theoretically advantageous in donor cell retention compared with the antegrade IC route, as retrograde IC injection would allow a longer time for the injected cells to interact with coronary vasculatures, in particular at postcapillary venules, by occluding the stem of the cardiac vein, compared with antegrade IC injection. In addition, retrograde IC injection enables a widespread cell delivery regardless of occluded arteries (i.e., into the normal, ischemic and infarcted areas) [68,69]. By contrast, antegrade IC cell injection after reopening the responsible coronary artery for treating acute MI might result in limited cell delivery due to impairment of coronary endothelial function, such as 'no-reflow' phenomena [81]. Although it was reported that cell retention is not significantly different between the antegrade and the retrograde cell injection [21], the protocol was much less optimized in retrograde IC injection. Appropriate optimization of the injection protocol, such as injection pressure, cell density or occlusion time, might yield different results.

iv. cell injection

Other possible routes for cell delivery into the heart include systemic iv. injection [82,83]. This method is much less invasive and more economical than other methods, and thus draws the interest of many scientists and clinicians. However, this systemic route via either peripheral vein, central vein or right/left atrium is highly likely to result in severely limited efficiency in delivering donor cells into the heart, as cells injected via this route are largely entrapped in other organs, such as the lung, prior to reaching the heart. Aicher *et al.* reported that iv. injection of radiolabeled EPCs of human origin into athymic nude rats following MI yielded only 2% of the total radioactivity retained in the heart, with 70% being retained in the liver and the spleen after 24–96 h [84]. This finding has been replicated by other studies using BMMNCs [85] or purified $CD34^+$ cells [86].

Success of this route is, therefore, dependent on the improvement of cell delivery efficiency to the heart. This may be achieved by amplifying the homing signals that prompt donor cells to effectively recruit/migrate to the host myocardium. Interestingly, it is reported that MSCs are preferentially accumulated in the failing heart after iv. injection [86–88], suggesting that efficacy of homing to the heart is donor-cell-type specific. In addition, the condition of the host myocardium, particularly the coronary endothelium, will be important for effective homing of donor cells into the heart. Importantly, Hare *et al.* reported 6-month results of a double-blind, placebo-controlled clinical study, in which allogeneic MSCs were intravenously injected, on treating acute MI postreperfusion, showing improvement in symptom and ejection fractions of the LV by this treatment [89]. Further investigations to understand and enhance homing signals in both donor cells and the host myocardium, which should be donor-cell-type specific, are essential for the future success of iv. cell injection.

Epicardial cell placement: the cell-sheet technique

Epicardial cell placement is an emerging method for cell delivery to the heart. This became practical since the development of the 'cell sheet' technique by Okano *et al.* [90] and Sawa *et al.* [91].

Principal concept of the cell-sheet method

It is now possible to generate scaffold-free cell sheets by using the thermoresponsive culture dishes (Cell Seed Inc., Tokyo, Japan), which are coated with a temperature-responsive polymer that is hydrophilic at 37°C, while hydrophobic below 25°C [92]. These dishes allow cells to attach to the bottom and grow at 37°C as usual. However, by decreasing the culture temperature to 25°C or below, the confluent cells are spontaneously detached from the dish, generating a scaffold-free cell sheet.

Donor cells contained in the cell sheet are advantageous as donors for cell transplantation over those in cell suspensions. Due to cell dissociation procedures, such as trypsinization, the cells prepared as cell suspensions will have suffered damages before transplantation; cell surface proteins, intercellular connections and underlying ECM can be damaged. By contrast, the donor cells prepared as the cell sheet preserved

cell surface proteins and ECM with maintained intercellular communications with the adjacent donor cells [93], assuring higher viability and functionality of donor cells.

The cell sheets, monolayer or multilayer, can be placed onto the epicardial heart surface to target the areas of interest. In addition to the preserved functionality of the cells, this method – epicardial placement of cell sheets – has been shown to cause less mechanical damage to both donor cells and host myocardium compared with other methods [94,95]. Furthermore, donor cell retention and subsequent therapeutic efficacies are known to be increased by this method compared with IM, IC and iv. injection [95].

Donor cell retention & survival following cell-sheet placement

It has been shown that the majority of donor cells that are transplanted by the cell-sheet technique retain on the epicardial surface, while only a small number of the transplanted cells are migrating or integrating into the host myocardium [96–98]. The initial donor cell retention rate after the cell-sheet technique is much higher compared with that after IC or IM injection [20,21,63,99].

Following epicardial placement of the cell sheets, the ECM and/or surface proteins of the cell sheets would generate firm attachment to the heart. This is thought to be the reason for the high retention rate after the cell-sheet method. Subsequent survival of the cells following cell-sheet transplantation has not been fully addressed. It was reported that transplanted cells surviving on the epicardial surface are supported by newly generated vascular networks from the host myocardium [99]. However, blood supply via such new vasculatures is likely to be insufficient and this might limit the long-term survival of the donor cells [100].

Therapeutic potential of the cell-sheet method in treating heart failure

It has been shown that the cell-sheet method using various types of adult stem/progenitor cells, including SMBs, MSCs and cardiac progenitor cells, induce functional recovery in heart failure [101,102]. The mechanism underlying this effect is believed to be the paracrine effects, in which donor cells secrete a specific group of protective factors into the host cardiac tissue to enhance recovery and healing and regenerating processes [95,96]. The magnitude of the paracrine effects has been suggested to be dependent upon the cell source and the number of surviving functional cells [96,103]. Therefore, the cell-sheet method, which can achieve an increased number of surviving donor cells in the heart, could theoretically achieve augmentation of the paracrine effects and, consequently, therapeutic outcome, compared with other cell-delivery methods [103]. There have been reports of a more than 30% improvement in ejection fraction of the LV by SMB cell-sheet transplantation in a chronic MI model, although there are no reports that appropriately compared recovery of cardiac function between the cell-sheet method and other cell-delivery methods [95,103].

Following a series of preclinical studies, this method has entered a clinical stage using SMBs in Japan [91]. In this Phase I clinical study, cell sheets generated by autologous SMBs were placed in patients who required mechanical support due to end-stage cardiac failure. The results are promising, although preliminary. The cell-sheet placement was feasible and safe, and improved cardiac function and structure in general, enabling successful weaning from the mechanical-assist device in some patients. Further large-scale, randomized clinical studies are warranted to prove the precise effect of this strategy.

Enhancing the cell-sheet method

Possible limitations of the cell-sheet method in achieving the maximal therapeutic potential include the poor vascular networks between the host myocardium and cell sheets, which will limit the survival of donor cells. Treatment of the cell sheet prior to the epicardial placement with heat shock or ischemic stimuli might upregulate protective factors in the cell sheet, enhancing the survival of donor cells. It has been shown that covering the cell sheets with the pedicle omentum flap, which possesses abundant vascular networks with angiogenic potentials, may be useful in supporting the transplanted cell sheet and enhancing its survival [100]. Concomitant injection of other cell types or substrates that carry angiogenic potentials into the target myocardial area could enhance generation of vascular network support for the cell sheets and might consequently enhance the therapeutic effects.

Conclusion & future perspective

Although randomized clinical studies have suggested that therapeutic benefits of cell transplantation therapy for treating cardiac failure may not be as substantial as expected in experimental studies, there are several promising strategies to further enhance therapeutic effects of this emerging treatment. The cell-delivery method is one of the most important targets for this aim.

Currently, there is no perfect method and, thus, donor cell type and cardiac pathology-specific choice of cell-delivery method would also be important.

Most adult stem cells, including MSCs, EPCs and SMBs, are likely to achieve the therapeutic effects via the paracrine effects, rather than differentiation to cardiomyocytes. For these cells, simple injection methods discussed above, such as IM, IC or cell-sheet methods, may be useful and subject to further enhancement. This is because paracrine effects can be induced even if donor cells do not necessarily form gap junctional intercellular communications with host cardiomyocytes. However, generation of new cardiomyocytes from donor cells is a major aim of cell transplantation therapy for the treatment of end-stage cardiac failure. To this end, cells that have established cardiomyogenic potency, including embryonic stem cells and induced pluripotent stem cells, are promising as donors, but these cell types require further consideration when choosing the cell-delivery route. New cardiomyocytes derived from these stem cells have to make appropriate intercellular communications – biochemically and electrically – with host cardiomyocytes. Otherwise, isolated new cardiomyocytes are unable to contribute to the global cardiac function and, furthermore, could be a source of arrhythmias. More substantial development, including tissue-engineering technologies, may be needed to transplant stem cell-derived cardiomyocytes into the heart.

One of the most important issues in this field that needs to be addressed is the development of clinically applicable *in vivo* cell-tracking methods [104]. Although functional and prognostic improvement is the principal goal of cell transplantation therapy for heart disease, assessment of the distribution and viability of transplanted cells after treatment would further clarify the efficacy and underlying mechanisms. Serial visualization of the transplanted cells after the treatment has been shown to be feasible by using nuclear imaging or MRI, although the cells need to be labeled by radioactives or genetic modification, respectively, prior to transplantation.

To conclude, there is no ideal cell-delivery method into the heart. Choice of cell-delivery method will be dependent upon the donor cell type and the nature of target cardiac pathology. Every combination that has been developed and studied has advantages and disadvantages. Appropriate comparison among the cell-delivery methods by preclinical or further large-scale, better organized clinical studies is warranted to further understand and improve the current cell-delivery methods, including the development of novel methods, leading to future clinical success of cell transplantation therapy for the treatment of heart failure.

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Executive summary

- There are four major cell-delivery routes into the heart for cell transplantation therapy treating heart disease: intramyocardial, intracoronary, intravenous and epicardial placement.
- Each cell-delivery route has potential advantages and disadvantages. The most suitable route should be taken in each patient in a tailor-made manner depending upon donor cell type and treating cardiac pathology.
- Further experimental and clinical investigations to further understand and refine each cell-delivery method are needed for the real success of cell transplantation therapy. The development of a novel route is also warranted.

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▪▪ **First-in-man study for cell-sheet placement for heart disease.**

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Sustained-Release Delivery of Prostacyclin Analogue Enhances Bone Marrow-Cell Recruitment and Yields Functional Benefits for Acute Myocardial Infarction in Mice

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Abstract

Background: A prostacyclin analogue, ONO-1301, is reported to upregulate beneficial proteins, including stromal cell derived factor-1 (SDF-1). We hypothesized that the sustained-release delivery of ONO-1301 would enhance SDF-1 expression in the acute myocardial infarction (MI) heart and induce bone marrow cells (BMCs) to home to the myocardium, leading to improved cardiac function in mice.

Methods and Results: ONO-1301 significantly upregulated SDF-1 secretion by fibroblasts. BMC migration was greater to ONO-1301-stimulated than unstimulated conditioned medium. This increase was diminished by treating the BMCs with a CXCR4-neutralizing antibody or CXCR4 antagonist (AMD3100). Atelocollagen sheets containing a sustained-release form of ONO-1301 (n = 33) or ONO-1301-free vehicle (n = 48) were implanted on the left ventricular (LV) anterior wall immediately after permanent left-anterior descending artery occlusion in C57BL6/N mice (male, 8-weeks-old). The SDF-1 expression in the infarct border zone was significantly elevated for 1 month in the ONO-1301-treated group. BMC accumulation in the infarcted hearts, detected by in vivo imaging after intravenous injection of labeled BMCs, was enhanced in the ONO-1301-treated hearts. This increase was inhibited by AMD3100. The accumulated BMCs differentiated into capillary structures. The survival rates and cardiac function were significantly improved in the ONO-1301-treated group (fractional area change 23±1%; n = 22) compared to the vehicle group (19±1%; n = 20; P = 0.004). LV anterior wall thinning, expansion of infarction, and fibrosis were lower in the ONO-1301-treated group.

Conclusions: Sustained-release delivery of ONO-1301 promoted BMC recruitment to the acute MI heart via SDF-1/CXCR4 signaling and restored cardiac performance, suggesting a novel mechanism for ONO-1301-mediated acute-MI heart repair.

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Competing Interests: The authors have read the journal's policy and have the following conflicts: Y. Sakai was an employee of Ono Pharmaceutical Co. Ltd., and a holder of the patent for ONO-1301 encapsulated in PLGA microspheres (patent numbers WO 2004/032965 and WO 2008/047863). There are no other patents, products in development, or modified products to declare. The other authors have declared that no competing interests exist. This does not alter the authors' adherence to all PLOS ONE policies on sharing data and materials.

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Introduction

Despite a number of medical and interventional treatments have been developed to treat acute myocardial infarction (AMI), the treatment for massive AMI has not been fully established. Myocardial infarction (MI) is a progressive disease, characterized by massive ischemic necrosis of the myocardial tissue and subsequent inflammation. This leads to cardiac remodeling that exacerbates the oxygen shortage in the surviving cardiac tissue. These pathological and functional deteriorations eventually cause end-stage heart failure. To delay the progression of heart failure, it is essential to suppress inflammation and fibrosis and to improve bloodflow supply in the injured myocardium consecutively. Recently, stromal cell-derived factor (SDF)-1 and its corresponding receptor CXCR4 have been shown to play prominent roles in homing of bone marrow cells (BMC) which promotes neovascularization and prevention of apoptosis via paracrine mechanism [1,2,3,4].

ONO-1301 (({5-[2-({[(1E)-phenyl(pyridin-3-yl)methylene]amino}oxy)ethyl]-7,8-dihydronaphthalen-1-yl}oxy)acetic acid) is a synthetic prostacyclin agonist. As it lacks the typical prostanoid

structure of a five-membered ring and an allylic alchol, ONO-1301 is chemically and biologically stable *in vivo*. In addition, thromboxane A2 synthethase is inhibited by ONO-1301, resulting in the promotion of endogenous prostacyclin synthesis. ONO-1301 has been reported to induce the production of endogenous hepatocyte growth factor (HGF) and vascular-endothelial growth factor (VEGF) in fibroblasts by stimulating cAMP production [5,6,7,8]. The administration of a slow-release form of ONO-1301 shows therapeutic potential, mainly due to the restoration of bloodflow in MI models of rat and swine and in a cardiomyopathic hamster [6,7,8]. The potential mechanism of the functional benefits of ONO-1301 mainly result from the enhanced secretion of growth factors, such as HGF and VEGF, which induce angiogenesis, restore bloodflow, and attenuate the progression of fibrosis. Recently we identified that ONO-1301 also upregulates SDF-1 secretion in the fibroblasts. Enhanced BMC homing in the MI heart by ONO-1301 therapy is attractive therapeutic modality. We thus hypothesized that ONO-1301 can induce BMC accumulation mediated by the upregulation of SDF-1 to elicit functional improvement in a mouse model of MI.

Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Osaka University (H23–123). All surgery was performed under sodium pentobarbital or isoflurane anesthesia, and all efforts were made to minimize suffering.

ONO-1301 and a slow-release form of ONO-1301 were purchased from ONO Pharmaceutical Co. Ltd. (Osaka, Japan) [7,8,9].

Migration Assay

Normal human dermal fibroblasts (NHDFs; Takara bio, Shiga, Japan) were cultured with or without ONO-1301 for 72 hours. The SDF-1 concentration in the culture supernatants was measured by ELISA (R&D systems, MN). BMCs were obtained from a green fluorescent protein (GFP)-transgenic mouse [C57BL/6-Tg(CAG-EGFP); Japan SLC, Inc., Shizuoka, Japan], and their migration toward the supernatants was assessed using a culture insert system (BD Falcon). The number of migrated BMCs was determined using fluorescence microscopy (Carl Zeiss, Göttingen, Germany).

Mouse AMI Model and Sheet Transplantation

An AMI model was generated by permanent ligation of the left anterior descending artery (LAD) in 10-15-week-old male C57BL/6N, BALB/cA, or BM-GFP chimera mice [10]. ONO-1301 microspheres and control microspheres were resuspended in saline at 10 mg/ml and added to atelocollagen sheets just before transplantation. Five minutes after the LAD ligation, atelocollagen sheets that included ONO-1301-containing microspheres (ONO-1301-treated group, n = 40) or empty microspheres (vehicle group, n = 40) were fixed onto the surface of the anterior left ventricular (LV) wall. The mice were euthanized 7, 21, and 28 days after the LAD ligation and ONO-1301 administration.

Assessment of BMC Homing

BMCs harvested from BALB/cA mice were labeled by Xenolight DiR (Caliper Life Sciences, MA) following the manufacturer's instructions and injected into the tail vein of BALB/cA mice after the MI and ONO-1301 treatment. On days 1 and 3, the whole-body imaging of the mice was measured by an *in vivo* imaging system (IVIS, Caliper Life Sciences).

Assessment of Cardiac Function and Survival

Cardiac function was assessed using an echocardiography system equipped with a 12-MHz transducer (GE Healthcare, WI) 4 weeks after MI and ONO-1301 treatment. The LV areas were measured, and LV fractional area change (FAC) was calculated as (LVEDA-LVESA)/LVEDA×100, where LVEDA and LVESA are the LV end-diastolic and end-systolic area, respectively.[10] The mice were housed in a temperature-controlled incubator for 28 days post-treatment to determine their survival.

Histological Analysis

Frozen sections (8 μm) of hearts were stained with antibodies against von Willebrand factor (vWF; Dako, Glostrup, Denmark) and CD31 (Abcam, UK). The secondary antibody was Alexa 546 goat anti-rabbit (Life Technologies, CA). Counterstaining was performed with 6-diamidino-2-phenylindole (DAPI; Life Technologies). The sections were also stained with isolectin (Life Technologies) following the manufacturer's instructions. To count GFP-positive cells, isolectin-positive cells, and CD31-positive capillary densities, 10 images were captured for each specimen. Capture and analysis were performed using Biorevo (Keyence, Japan). To analyze the myocardial collagen accumulation, heart sections were stained with Masson's trichrome. The collagen volume fraction in the peri-infarct area was calculated.

Quantitative Real-time PCR

The total RNA was isolated from the peri-infarct area using the RNeasy Mini Kit and reverse transcribed using Omniscript Reverse transcriptase (Qiagen, Hilden, Germany). Quantitative PCR was performed with a PCR System (Life Technologies). The expression of each mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers and probes are shown in Table S1 in File S1.

Statistical Analysis

Data are expressed as the mean ± SEM. The data distributions were checked for normality. Comparisons between 2 groups were made using the Student's *t*-test. For comparisons among 3 or more groups, one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test were used. The survival curves were prepared using the Kaplan-Meier method and compared using the log-rank test. All *P*-values are two-sided, and values of $P<0.05$ were considered to indicate statistical significance. Statistical analyses were performed using the StatView 5.0 Program (Abacus Concepts, Berkeley, CA) and Statcel2 (The Publisher OMS Ltd., Saitama, Japan).

An expanded Methods section can be found in the online-only in File S1.

Results

ONO-1301 Enhanced BMC Migration via SDF-1/CXCR4 Signaling

The effect of ONO-1301 on the SDF-1 secretion by NHDFs was evaluated by ELISA. As shown in Fig. 1A, the SDF-1 concentration in the NHDF culture supernatants increased in an ONO-1301 concentration-dependent manner. The SDF-1 concentration in the culture supernatant of 1000 nM ONO-1301-treated cells was significantly greater than that of cells cultured in

the absence of ONO-1301 (Fig. 1A). To investigate the BMC migration toward ONO-1301-treated NHDF conditioned medium, a migration assay was performed using a modified Boyden chamber with 8-μm pores. The number of migrated BMCs was significantly greater in the conditioned medium of cells treated with 100 and 1000 nM ONO-1301 compared to that of cells treated with 0 and 10 nM ONO-1301. The BMC migration to the 1000 nM ONO-1301 conditioned medium was diminished by treating the BMCs with a CXCR4-neutralizing antibody or CXCR4 antagonist (AMD3100) (Fig. 1B, C).

SDF-1-mediated BMC Accumulation in the ONO-1301-treated Infarcted Hearts

The effect of ONO-1301 on SDF-1 expression in the infarcted hearts was evaluated by quantitative RT-PCR. Twenty-eight days after treatment, the SDF-1 expression in the border area of the ONO-1301-treated heart was significantly greater than that in the vehicle-treated heart (Fig. 2A). The HGF and VEGF expressions were also increased by ONO-1301 treatment (Fig. 2B, C). After LAD occlusion, ONO-1301 treatment, and intravenous injection of labeled BMCs, the BMC accumulation in the infarcted heart was evaluated by an in vivo imaging system. The proportion of BMCs in the heart showed a trend toward upregulation, dependent on the dose of ONO-1301 (Fig. 2D). Hearts treated with 100 mg ONO-1301/kg body weight showed significantly more accumulated BMCs than those treated with 0 or 10 mg ONO-1301. In 100 mg/kg ONO-1301-treated hearts, CXCR4 antagonization significantly decreased the BMC accumulation (Fig. 2D). To identify the recruited BMCs *in vivo*, the acute MI model was prepared using chimera mice by transplanting GFP-expressing bone marrow into irradiated C57BL/6 mice. The BMCs of the C57BL/6 transplant recipients were largely replaced by GFP-expressing BMCs (91.8+/−4.3%, figure S1 in File S1). The single-organ analyses using GFP-BM chimera mouse at day 7 also showed increased BMC accumulation in the ONO-1301-treated myocardium (figure S2 in File S1).

Differentiation of BMCs in the Infarcted Myocardium

Seven days after MI and ONO-1301 administration to BM-GFP chimera mouse, BMCs were dramatically accumulated in both the infarcted area and the atelocollagen sheet (Fig. 3A, B). Some of the BMCs formed tube-like structures and displayed von Willebrand factor expression (Fig. 3C, D). Isolectin staining showed that a greater percentage of isolectin-positive BMCs accumulated in the myocardium in the ONO-1301-treated (O) group than in the vehicle (V) group (Fig. 3E, F). We also evaluated small blood vessels by CD31 immunostaining. The density of small vessels was greater in the O group than in the V group (Fig. 3G). Immunohistochemical analysis of Connexin 43 and smooth muscle actin, cardiac-lineage and cardiac fibroblast markers, respectively, was also conducted at 3 months, but no co-expression

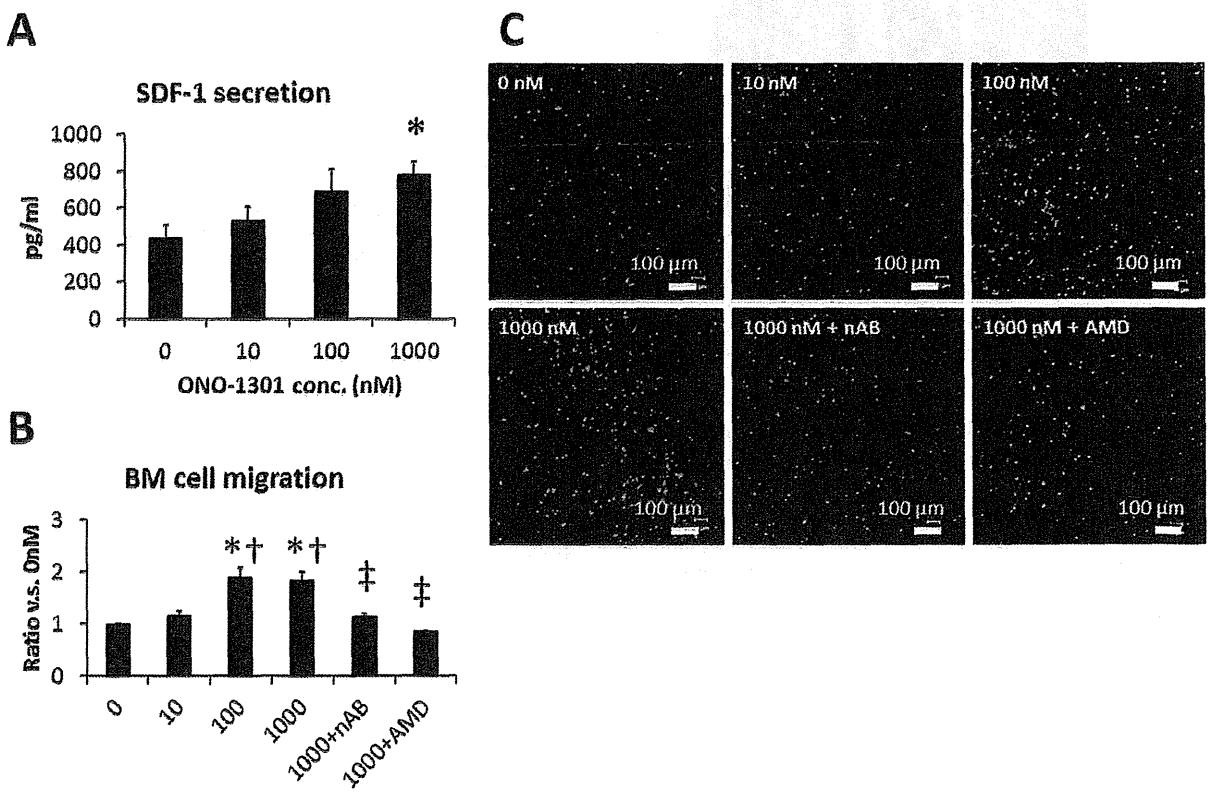

Figure 1. ONO-1301 enhanced SDF-1 secretion and BMC migration via SDF-1/CXCR4 signaling *in vitro*. NHDFs were stimulated with ONO-1301 for 72 hours, then the SDF-1 concentration in the culture medium was determined by ELISA (n = 3 each, *P<0.05 vs. 0 nM). A) Number of BMCs that migrated toward the conditioned medium from ONO-1301-stimulated-NHDFs (0, 10, 100, or 1000 nM ONO-1301, n = 6; 1000 nM+nAB or 1000 nM+AMD, n = 3). *P<0.05 vs. 0 nM, †P<0.05 vs. 10 nM, ‡P<0.05 vs. 1000 nM, §P<0.05 vs. SDF-1. nAB, CXCR4-neutralizing antibody; AMD, CXCR4 antagonist AMD3100. B) Representative pictures of BMCs that had migrated to the medium from ONO-1301-stimulated BMCs. Green, BMCs.
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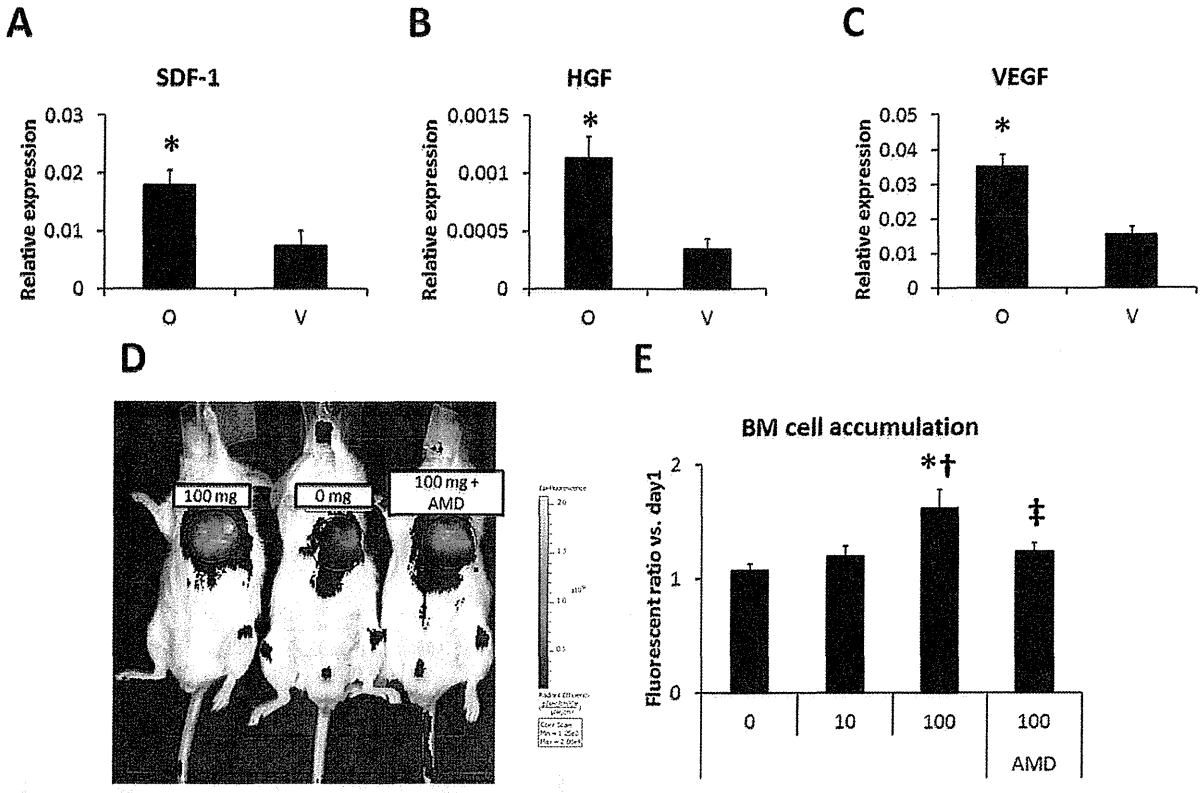

Figure 2. ONO-1301 enhanced SDF-1 secretion and BMC migration via SDF-1/CXCR4 signaling after MI. A–C) The SDF-1, HGF, and VEGF expression at the border zone of the infarcted area was measured by quantitative RT-PCR. The expression levels of these cytokines were higher in the ONO-1301-treated (O) group compared to the vehicle (V) group. (O group, n = 7; V group, n = 7–8; *$P<0.05$ vs. V group). The expression relative to GAPDH is shown. D) BMC migration to ONO-1301-treated infarcted myocardium was evaluated using IVIS. Representative picture of IVIS at day 3. Left: 100 mg/Kg, Center: 0 mg/Kg, Right: 100 mg/Kg+AMD3100 (AMD). E) The number of accumulated BMCs was greater in the 100 mg/kg ONO-1301-treated infarcted heart compared to the 0 and 10 mg/kg ONO-1301-treated infarcted heart. When BMCs treated with AMD were injected, the BMC accumulation decreased in the 100 mg/Kg ONO-1301-treated infarcted heart compared with the untreated-BMC-injected heart (0 mg/Kg, n = 4; 10 mg/Kg, n = 8; 100 mg/Kg, n = 5; 100 mg/Kg+AMD3100, n = 4; *$P<0.05$ vs. 0 mg/Kg, †$P<0.05$ vs. 10 mg/Kg, ‡$P<0.05$ vs. 100 mg/Kg).
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of GFP with either of these markers was observed (figure S3 in File S1).

Therapeutic Effects of ONO-1301 Administration on Cardiac Performance, Survival, and LV-remodeling at 4 Weeks Post-MI

ONO-1301 was detected in the plasma of blood samples from the ONO-1301-treated group 3 weeks after treatment (figure S4 in File S1). The cardiac functions in the MI mice with and without following ONO-1301 treatment were evaluated. Mortality was substantial until 14 days post-LAD ligation in the vehicle group, and similar mortality levels were observed with non-treated MI mice [11]. In contrast, in the ONO-1301-treated group, there was little mortality 7 days after MI, and thus a difference in survival (Fig. 4A). Cardiac performance was evaluated by 2D echocardiography 4 weeks after implantation. The LVEDA was smaller in the ONO-1301-treated group than in the vehicle group, but the difference was not significant. In contrast, the LVESA was significantly smaller, and the LVFAC was significantly greater, in the ONO-1301-treated group than in the vehicle group (Fig. 4B). In the histological analysis, the vehicle group showed a typical MI with a large anterior LV scar and dilatation of the LV cavity. By comparison, the LV of the ONO-1301-treated group was less dilated, and the anterior wall was thicker (Fig. 4C, D). The infarcted area and percent fibrosis were significantly smaller in the ONO-1301-treated than in the vehicle-treated group (Fig. 4C, E–G).

Discussion

Here, we showed that ONO-1301 promotes BMC accumulation in the injured myocardium. *In vitro*, ONO-1301 enhanced SDF-1 expression, and BMC migration was greater to conditioned medium obtained from ONO-1301-stimulated cells. The enhanced migration was diminished by blocking SDF-1/CXCR4 signaling. Consistent with the *in vitro* experiments, ONO-1301 enhanced the SDF-1 expression of myocardial tissue. High ONO-1301 accelerated the BMC accumulation after MI in a SDF-1/CXCR4-dependent manner. Some BMCs in the infarcted myocardium differentiated into capillary structures within 7 days. Furthermore, the sustained-release delivery of ONO-1301 in the infarcted myocardium also led to functional improvements following MI. Our data suggest that ONO-1301 is a novel inducer of BMC recruitment, and that ONO-1301 treatment may be a promising therapeutic strategy for the clinical treatment of MI.

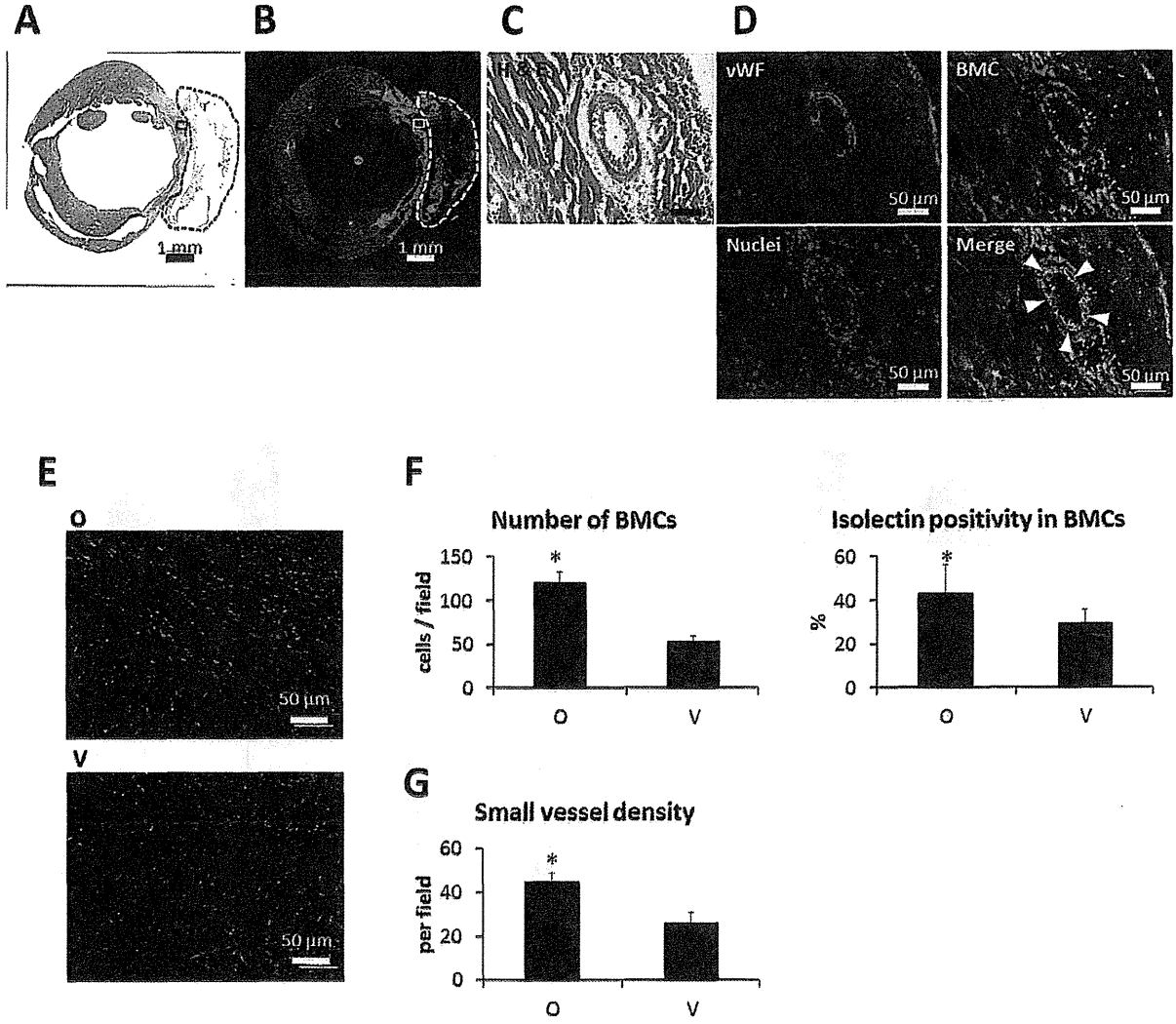

Figure 3. BMCs differentiated into capillary structures in the infarcted area after MI and ONO-1301 treatment. Representative macro image of H and E staining seven days after MI and ONO-1301 treatment. The transplanted sheet is enclosed by a dashed line. A) Serial section of A. The BMCs displayed GFP. B) High-magnification image of the boxed region in A. C) Serial section of C. Arrowheads indicate vWF-expressing BMCs. Red indicates vWF; green, BMCs; and blue, nuclei. D) Representative images of isolectin-stained BMCs seven days after MI and ONO-1301 treatment. E) BMC accumulation and percentages of isolectin-positive BMCs. The number of BMCs that accumulated in the infarcted myocardium was greater in the ONO-1301-treated (O) group than in the vehicle (V) group. The percentage of isolectin-positive BMCs was also greater in the O group than in the V group. *$P<0.05$ vs. V group. F) Small vessel density. Small vessels were detected by CD31 immunostaining. The density of small vessels in the O group was greater than in the V group. *$P<0.05$ vs. V group.
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It is difficult to understand the whole mechanism underlying the functional improvements induced by ONO-1301. It was already reported that ONO-1301 enhances the expression of angiogenic factors HGF and VEGF, leading to angiogenesis and the suppression of fibrosis progression [7,8,9]. In this study, we discovered an alternative mechanism for ONO-1301's therapeutic efficacy in the acute MI mouse, in which the upregulation of SDF-1 promotes BMC accumulation. Stem-cell recruitment and homing are regulated by the interplay of cytokines, chemokines, and proteases. In particular, the SDF-1/CXCR4 axis is central for the mobilization of stem cells from the bone marrow and their homing to ischemic tissues [12]. In the case of ischemic insult, SDF-1 is released by the injured tissue and stimulates the mobilization of progenitor cells from the bone marrow [1,13]. Furthermore, prostaglandins have been reported to facilitate BMC mobilization via upregulation of CXCR4 expression [14,15]. In our experimental setting, ONO-1301 was detected from peripheral blood samples 3 weeks after treatment (Fig. S4 in File S1), suggesting that ONO-1301 may similarly act on the bone marrow to promote the BMC mobilization. Thus, BMC recruitment in the injured myocardium may be enhanced by the upregulation of SDF-1 in cardiac fibroblasts and by the direct upregulation of CXCR4 in BMCs located in the bone marrow. In addition, recent reports show the possibility of endogenous regeneration in the injured heart, including proliferation of postnatal cardiomyocytes and cardiac stem cells [16,17,18,19]. While we were unable to

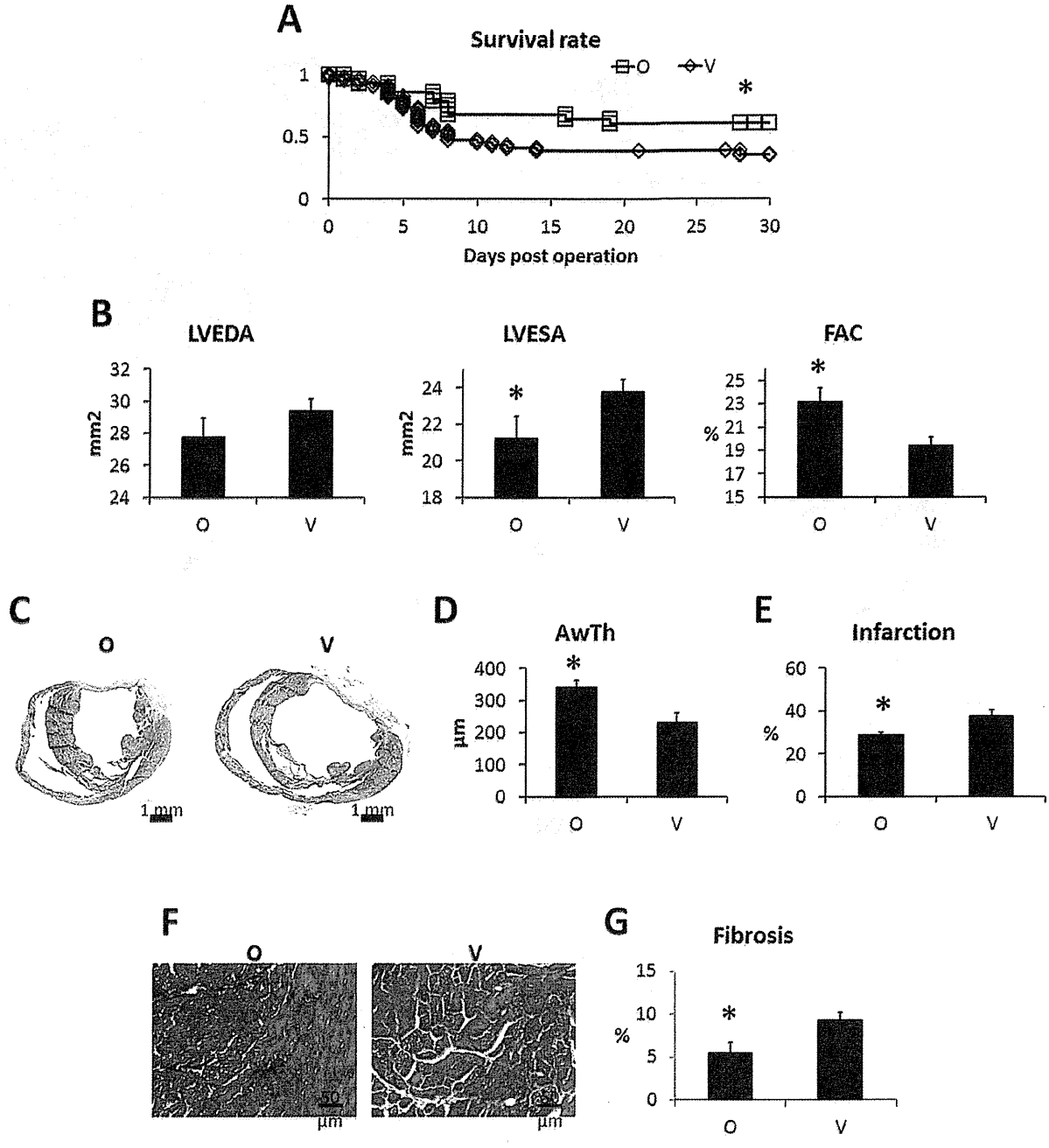

Figure 4. ONO-1301 treatment improved the cardiac performance and survival rate after MI. Survival rates after treatment. The ONO-1301-treated (O) group (n = 33) showed significantly better survival than the vehicle (V) group (n = 48). *P<0.05 vs. V group. A) Evaluation of cardiac performance 4 weeks after treatment. In the O group, the LVESA was smaller, and the FAC was significantly higher compared to the V group (O group, n = 22; V group, n = 20; *P<0.05 vs. V group). B) Representative macro images from each group. C) Quantification of anterior wall thickness. Anterior wall thickness was significantly thicker in the O group (n = 6) compared to the V group (n = 4). *P<0.05 vs. V group. D) Quantification of percent infarction. Infarction was significantly smaller in the O group (n = 6) compared to the V group (n = 4). *P<0.05 vs. V group. E) Representative Masson trichrome staining images at the border zone. F) Quantification of fibrosis. Fibrosis at the border zone was significantly smaller in the O group (n = 6) compared to the V group (n = 4). *P<0.05 vs. V group.
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detect newly-generated cardiomyocytes derived from BMCs in this study, it would be interesting to evaluate the possibility of cardiomyogenesis involving other cell types.

We observed massive BMC accumulation 7 days after MI, including in the infarcted ventricular wall, where they provided structural support in place of the necrotic cardiomyocytes. The

BMCs recruited into the infarcted myocardium may contain various kinds of somatic stem cells, such as endothelial progenitor cells [20], bone marrow-derived stem cells [21], and bone marrow mononuclear cells [2], which have potent therapeutic effects in heart failure [22]. Furthermore, bone marrow-derived mesenchymal stem cells secrete prostaglandin [23], which may act like ONO-1301 and amplify the effects of the ONO-1301-mediated therapy. Kawabe et al. clearly showed that prostaglandin facilitates the recruitment of endothelial progenitor cells [24]. Although further analysis is needed, the enhanced accumulation of BMCs may predispose the damaged heart tissue to better restoration following MI.

Many reports have shown that granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) also induce BMC mobilization, with therapeutic effects in animal models [25]. However, G-CSF therapy in unselected patients with acute MI did not lead to functional improvements beyond those achieved with conventional therapy. In addition, the administration of GM-CSF in cancer patients has been shown to transiently increase the LV end-systolic dimensions and decrease cardiac contractility [25,26]. The lack of efficacy of G-CSF therapy in clinical trials may be due, at least in part, to its poor initiation and duration; such therapies are likely to be most beneficial during the early phase after acute MI. Although conventional prostacyclin and its analogs are chemically and biologically unstable, ONO-1301 is a long-acting prostacyclin agonist that exerts stable effects *in vivo*, because it lacks a prostanoid structure. Furthermore, we used a slow-release form of ONO-1301, made by polymerizing it with poly-lactic and glycolic acid; this ONO-1301 could still be detected in the blood 3 weeks after its administration (figure S4 in File S1).

Furthermore, in our *in vitro* analysis, although we used normal human dermal fibroblasts to examine the SDF-1/CXCR-4-dependent BMC migration, the reactivity to ONO-1301 stimulation will differ depending on the cell type. For example, the G-CSF expression was upregulated in some kinds of cells (unpublished data). Thus, together with the upregulation of multiple beneficial cytokines such as HGF and VEGF, because of the longer duration of its activity, ONO-1301 may be more potent than conventional protein-based therapies.

Our data showed that ONO-1301 treatment was a potent inducer of BMC homing. Of the BMCs that accumulated in the infarcted myocardium, 43 percent expressed isolectin, an endothelial cell marker, but the other BMCs had a fibroblastic morphology, and did not express cardiac-lineage or cardiofibroblast markers (Figure S3 in File S1). ONO-1301 administration resulted in the attenuation of cardiac dysfunction, with enhanced BMC accumulation. Further study is required to elucidate the mechanism, but we speculate that paracrine effects of factors released by the BMCs play pivotal roles in the therapeutic efficacy, rather than the transdifferentiation of the BMCs into the cardiac or vascular lineage. The effect of cardioprotective and angiogenic factors secreted by the accumulated BMCs and the direct stimulation of ONO-1301 itself may synergistically increase the angiogenesis and cardioprotection, leading to improved therapeutic results.

In summary, ONO-1301 may be a powerful, long-acting activator of multiple cytokines. In particular, SDF-1 may enhance the BMC accumulation in a SDF-1/CXCR4-signaling-dependent manner, leading to an attenuation of the cardiac dysfunction following MI. Our findings suggest that the method involving a sustained release of ONO-1301 may be adapted as a novel drug delivery system for treating heart failure.

Supporting Information

File S1.
(DOCX)

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Author Contributions

Conceived and designed the experiments: YI SM Y. Sawa. Performed the experiments: YI KI NS. Analyzed the data: YI AS. Contributed reagents/materials/analysis tools: Y. Sakai. Wrote the paper: YI SM SF Y. Sawa.

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Improvement of Cardiac Stem Cell-Sheet Therapy for Chronic Ischemic Injury by Adding Endothelial Progenitor Cell Transplantation: Analysis of Layer-Specific Regional Cardiac Function

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Running title: Combined CSC sheet and EPC Therapy for ICM

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ABSTRACT

BACKGROUND: The transplantation of cardiac stem cell sheets (CSC sheets) is a promising therapeutic strategy for ischemic cardiomyopathy, although potential ischemia in the transplanted area remains a problem. Injected endothelial progenitor cells (EPCs) can reportedly induce angiogenesis in the injected area. We hypothesized that concomitant CSC sheet transplantation and EPC injection might show better therapeutic effects for chronic ischemic injury model than the transplantation of CSC sheets alone.

METHODS: Scaffold-free CSC-sheets were generated from human c-kit-positive heart-derived cells. A porcine chronic ischemic injury model was generated by placing an ameroid constrictor around the left coronary artery for 4 weeks. The animals then underwent a sham operation, epicardial transplantation of CSC sheet over the ischemic area, intramyocardial injection of EPCs into the ischemic and peri-ischemic area, or CSC-sheet transplantation plus EPC injection. The efficacy of each treatment was then assessed for 2 months.

RESULTS: Speckle-tracking echocardiography was used to dissect the layer-specific regional systolic function by measuring the radial strain (RS). The epicardial RS in the ischemic area was similarly greater after treatment with the CSC-derived cell-sheets alone (19±5%) or in combination with EPC injection (20±5%) compared with the EPC only (9±4%) or sham (7±1%) treatment. The endocardial RS in the ischemic area was greatest after the combined treatment (14±1%), followed by EPC only (12±1%), compared to the CSC only (11±1%) and sham (9±1%) treatments. Consistently, either epicardial CSC-sheet implantation or intramyocardial EPC injection yielded increased capillary number and reduced cardiac fibrosis in the ischemic epicardium or endocardium, respectively. Concomitant EPC injection induced the migration of transplanted CSCs into the host

myocardium, leading to further neovascularization and reduced fibrosis in the ischemic endocardium, compared to the CSC-sole therapy.

CONCLUSION: Transplantation of CSC-sheets induced significant functional recovery of the ischemic epicardium, and concomitant EPC transplantation elicited transmural improvement in chronic ischemic injury.

Key-words: Cardiac stem cell, Endothelial progenitor cell, Chronic ischemic injury, Strain imaging, Left ventricular remodeling

BACKGROUND

Transplantation of somatic tissue-derived stem cells has been shown to be a feasible, safe, and potentially effective treatment for advanced cardiac failure in clinical settings (6,32). In particular, cardiac stem cells (CSCs), represented by c-kit-positive cells in the myocardium, can play a central role in healing the damaged myocardium, through their direct differentiation *in situ*, the recruitment of circulating stem/progenitor cells, or the paracrine release of cardioprotective factors (9,12,30). CSC transplantation is therefore considered a promising treatment for advanced cardiac failure, although the optimal method for cell delivery into the heart is still under debate (7).

The transplantation of scaffold-free cell sheets was shown to enhance the retention and survival of the transplanted cells and to minimize the risks of cell-delivery-related myocardial damage that leads to arrhythmogenicity, thus showing good therapeutic potential (5,20,33). However, concerns remain regarding the integration of the transplanted cells into the myocardium, which would have a direct impact on regional cardiac function, and the

potential for ischemia in the transplanted cell sheet, which would limit its therapeutic potential. On the other hand, endothelial progenitor cells (EPCs) have been shown to induce neo-angiogenesis in the ischemic/infarcted myocardium and to activate residential CSCs to enhance healing and/or regeneration of the damaged myocardium (11,13,31). The intramyocardial injection of EPCs is thus another promising treatment for enhancing myocardial regeneration and possibly supporting the cellular function of transplanted CSCs (16).

We thus hypothesized that CSC transplantation by the cell-sheet technique might induce cardiomyogenic differentiation *in situ*, reverse left ventricular (LV) remodeling, and improve functional recovery in ischemic injury model and that these therapeutic effects might be enhanced by the concomitant transplantation of EPCs, which could have different effects on the damaged myocardium from CSCs.

Several lines of evidence suggested that region-specific, especially layer-specific, LV function assessed by recently-developing modalities may be superior to globally-measured ejection fraction (EF) in predicting myocardial recovery after a wide range of medical and surgical treatment (3,14). Here we used a porcine chronic ischemic injury model to dissect the layer-specific functional effects of these two types of cell transplantation.

METHODS

All human and animal studies were carried out with the approval of the institutional ethical committee. Human samples were collected under written informed consent. The investigation conforms to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health Publication No. 85-23, revised 1996). All experimental procedures and evaluations were carried out in a blinded manner.

Isolation and cultivation of c-kit-positive cells from human cardiac tissue

Human normal right atrial tissues were obtained from a 53-year-old female patient with dilated cardiomyopathy at Osaka University Hospital. The isolation method was as published recently (6). Briefly, after dissecting fat and fibrous tissue, the sample were cut into small pieces (< 1 mm^3) and suspended in 8 ml Ham's F12 medium (Wako Pure Chemical Inc., Osaka, Japan) containing 0.2% collagenase (17454, Serva Electrophoresis, Heidelberg, Germany). After digestion, cells were plated in culture dishes (353003, BD Falcon, Franklin Lakes, NJ) containing Ham's F12 supplemented with 10% FBS (SH30406.02, Hyclone, Thermo Scientific, Waltham, MA), 10ng/ml recombinant human basic fibrobrast growth factor (bFGF) (100-18B, PeproTech, Rocky Hill, NJ), 0.2 mM L-Glutathione (G6013, Sigma Aldrich, St Louis, MO) and 5 mU/ml erythropoietin (E5627-10UN, Sigma Aldrich). Subsequently, cells were expanded and subjected to fluorescence activated cell sorting (FACSAria) with antibody (CD117(AC126)-PE, 130-091-735, Miltenyi Biotec, Bergisch Gladbach, Germany) to obtain C-Kit positive CSCs. The sorted c-kit-positive CSCs were cultured until the fifth passage in medium containing Ham's F-12, 10% fetal bovine serum